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(54) Title: METHODS OF REGULATING IL-12 PRODUCTION BY ADMINISTERING CCR5 AGONISTS AND ANTAGONISTS

(57) Abstract: The present invention provides a method for increasing IL-12 production in a cell, comprising administering a CCR5 agonist to the cell. The invention also provides a method for decreasing IL-12 production in a cell, comprising administering a CCR5 antagonist to the cell. Further provided by this invention is a method for treating a disease associated with increased IL-12 production in a subject, comprising administering to the subject a CCR5 antagonist in an amount effective in reducing the disease-associated effect of IL-12, thereby treating the disease associated with increased IL-12 production. Also provided by this invention is a method for increasing IL-12 production in a subject, comprising administering and effective amount of a CCR5 agonist to the subject.

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METHODS OF REGULATING IL-12 PRODUCTION BY ADMINISTERING CCR5 AGONISTS AND ANTAGONISTS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to methods for increasing IL-12 production in a cell by administering CCR5 agonists and to methods for decreasing IL-12 production in a cell by administering CCR5 antagonists. The invention also relates to methods for increasing IL-12 production in a subject by administering CCR5 agonists and to methods for decreasing IL-12 production in a subject by administering CCR5 antagonists.

BACKGROUND ART

The activation of dendritic cells (DC) and their mobilization to secondary lymphoid organs is thought to be a crucial step in the initiation of adaptive immunity (1). Interleukin (IL)-12 in an important product of certain subpopulations of activated DC. This cytokine directs the selective development of Th1 responses and cellular immunity to infectious agents (2). However, the microbial signals that drive IL-12 synthesis by DC *in vivo* are poorly defined.

Although chemokines have a well-defined function in stimulating cell migration, they have not been thought to play a role in the induction of cytokines. Although CCR5 is known to be a major co-receptor for HIV-1 infection (8,9), its normal functions in the immune response are poorly understood. While patients with the CCR5 deletion mutation (Δ32) exhibit enhanced resistance to HIV, they so far have not been reported to display altered susceptibility to other unrelated infections (9,10). On the other hand, CCR5 deficient mice have been shown to display lowered resistance to *Cryptococcus neoformans* (11) and *Listeria monocytogenes* (12) as well as decreased IFN-γ production in response to infection with *Leishmania donovani* (13). Moreover,

CCR5-deficient animals show partial resistance to LPS-induced endotoxemia as well as decreased IL-1β, IL-6 and GM-CSF production by LPS-stimulated macrophages (12).

What has been heretofore unidentified has been a pathway by which the induction of chemokines can furnish an upstream signal for both cell mobilization and activation and as such, provide a primary step in the initiation of cell-mediated immunity to intracellular pathogens. The present invention overcomes previous shortcomings by establishing the existence of such a pathway, thereby providing for methods of regulating IL-12 production with agonists and antagonists of CCR5.

SUMMARY OF THE INVENTION

The present invention provides a method for increasing IL-12 production in a cell comprising administering a CCR5 agonist to the cell.

The invention also provides a method for decreasing IL-12 production in a cell comprising administering a CCR5 antagonist to the cell.

Further provided by this invention is a method for treating a disease associated with increased IL-12 production in a subject comprising administering to the subject a CCR5 antagonist in an amount effective in reducing the disease-associated effect of IL-12 thereby treating the disease associated with increased IL-12 production.

Also provided by this invention is a method for increasing IL-12 production in a subject comprising administering an effective amount of a CCR5 agonist to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-D. IL-12 production in mice pre-treated with Met-RANTES or genetically deficient in CCR5. The frequencies of IL-12p40$^+$CD11c$^+$CD8α^- determined by FACS (Figs. 1A and 1B) and spontaneous IL-12p40 secretion determined by ELISA (Figs. 1C and 1D) were analyzed in overnight cultures of splenocytes obtained from C57Bl/6 (Figs. 1A and 1C) or from CCR5 deficient mice (Figs. 1B and 1D) 6hr after PBS or STAg injection. In Figs. 1A and 1C, animals were pre-inoculated i.p. with Met-RANTES, human RANTES 30 min before *in vivo* microbial stimulation. The data shown are the means and SD of triplicate measurements performed on pools of spleen cells from five mice each (Figs. 1A and 1C) or of individual assays performed on five mice per group (Figs. 1B and 1D). B6129F2/J mice gave tissue and *in vitro* responses indistinguishable from those obtained with C57Bl/6 mice in the above experiments.

Figures 2A-D. Partial role of CCR5 in STAg-induced IL-12 production by DC *in vitro*. IL-12p40 production was measured in spleen cell culture supernatants 24hr after stimulation with STAg (1μg/ml). Fig. 2A shows the response of a LOD spleen cell fraction from C57Bl/6 mice in the presence of different concentrations of Met-RANTES antagonist while Figs. 2B and 2C compare the response of unfractionated spleen cells (Fig. 2B) or purified CD8α^+ DC (Fig. 2C) from B6129F2/J control with that of CCR5 deficient mice exposed to the same STAg stimulus. Fig. 2D demonstrates the inhibitory effect of different doses of PTx on STAg induced IL-12 production from LOD spleen cells obtained from C57Bl/6 mice.

Figures 3A-D. CCR5 ligands induce IL-12 production by splenic CD8α^+ DC *in vitro*. IL-12p40 was measured in culture supernatants of splenocytes or FACS-purified DC from naïve C57Bl/6 or CCR5 deficient mice 24 hr after *in vitro* stimulation with different chemokines. Fig. 3A shows the response of whole spleen cells to murine MIP-1β (200ng/ml) and Fig.3B the response of purified CD11c$^+$CD8α^+ or CD11c$^+$CD8α^- DC to the same stimulus. Fig. 3C compares the response of purified

CD8α⁻ DC to increasing concentrations of murine MIP-1α, MIP-1β, RANTES, MCP-1 and human MIP-5 while Fig. 3D demonstrates the effects of different doses of *Pertussis* toxin (PTx) on MIP-1β induced IL-12 production by a LOD fraction of spleen cells. Splenocytes from LPS-hyporesponsive C3H/HeJ mice displayed the same responsiveness to recombinant chemokines as the C57Bl/6 derived cells used above.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the examples included therein.

Before the present compounds and methods are disclosed and described, it is to be understood that this invention is not limited to specific proteins or specific methods. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

The present invention provides a method for increasing IL-12 production in a cell, comprising administering a CCR5 agonist to the cell.

As described herein, a "cell" of this invention can include any cell type, cancerous or noncancerous, that is capable of expressing CCR5, either endogenously or due to transduction of an exogenous nucleic acid encoding CCR5 and can be affected by the expression of a CCR5 gene or by the activity of CCR5. For example a cell of this invention can be, but is not limited to, a dendritic cell.

The term "agonist" as used herein refers to or describes a molecule which is capable of, directly or indirectly, substantially inducing, promoting or enhancing CCR5-mediated biological activity . Examples of CCR5 agonists include, but are not limited to, RANTES, MIP-1α, MIP-1β, MIP-5 and MCP-3, as well as any other CCR5 agonists now know or later identified to bind CCR5 or induce CCR5-mediated activity. The agonists of this invention can be administered to a cell or subject as a single agonist or as multiple different agonists in any combination.

The term "CCR5-mediated biological activity" as used herein refers to any activity mediated by CCR5, such as cell migration, cytokine synthesis activity or any other activity occurring as a result of a direct or indirect interaction of a substance with CCR5.

The invention further contemplates a bioassay for identifying agonists of CCR5-mediated activity, comprising contacting cells which express CCR5 with a test compound to be identified as an agonist of CCR5-mediated activity (e.g., growth factors, cytokines, chemokines, antibodies etc.), and measuring production of IL-12 by various assays, such as, for example, RT-PCR amplification or immunodetection, as are described herein and as are well known in the art, whereby an increase in IL-12 production, as compared to the amount of IL-12 production in control cells which are not exposed to the test compound, identifies a CCR5 agonist. To confirm that a test compound causes an increase in IL-12 production via CCR5, the test compound can be administered to CCR5 deficient cells, as described in the Examples herein. If no increase in IL-12 production in the CCR5 deficient cells is observed with the same test compound that caused an increase in IL-12 production in CCR5 expressing cells, the test compound is identified as a CCR5 agonist. Confirmation of agonist activity can also be achieved by exposing cells expressing CCR5 to the test compound in the presence of a CCR5 antibody or other CCR5 antagonist. If a dose dependent decrease in IL-12 production is observed under these conditions, the test compound is identified as a CCR5 agonist.

Numerous methods are available in the art for assaying the amount of cytokine (i.e., IL-12), and chemokine secretion from tissue obtained from a variety of sources, such as lamina propia, lymph nodes, spleen, liver, skin, lungs, joints, CNS and other tissues. These methods include, but are not limited to, tissue staining, FACS, ELISA, PCR, reverse-transcriptase-polymerase chain reaction and ELISPOT, Northern blots, Southern blots, and Western blots (16).

The present invention further provides a method for decreasing IL-12 production in a cell, comprising administering a CCR5 antagonist to the cell.

The term "antagonist" as used herein refers to or describes a molecule which is capable of, directly or indirectly, substantially counteracting, reducing or inhibiting CCR5-mediated biological activity. Examples of CCR5 antagonists include, but are not limited to, Met-RANTES, AOP-RANTES, LD-478, viral MIP-II and monoclonal or polyclonal antibodies against CCR5, as well as any other agents now known or later identified to block the binding of CCR5 to its agonists or ligands or to inhibit CCR5-mediated activity. The antagonists of this invention can be administered to a cell or subject as a single antagonist or as multiple different antagonists in any combination.

The invention also contemplates a bioassay for identifying a CCR5 antagonist comprising exposing cells *in vitro* to STAg in the presence of a test compound and measuring IL-12 production, whereby a dose dependent inhibition of IL-12 production by the cells identifies a test compound as a CCR5 antagonist.

The present invention further provides a method for treating a disease associated with increased IL-12 production in a subject, comprising administering to the subject a CCR5 antagonist in an amount effective in reducing IL-12, thereby treating the disease associated with increased IL-12 production.

Diseases associated with increased IL-12 include, but are not limited to, autoimmune diseases, inflammatory diseases, graft-versus-host disease (GvH) and transplantation rejection.

As used herein, "autoimmune disease" describes a disease state or syndrome whereby a subject's body produces a dysfunctional immune response against the subject's own body components, with adverse effects. This may include production of B cells which produce antibodies with specificity for all antigens, allergens or major histocompatibility (MHC) antigens, or it may include production of T cells bearing receptors that recognize self-components and produce cytokines that cause inflammation. Examples of autoimmune diseases can include, but are not limited to, ulcerative colitis, Crohn's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, pernicious anemia, autoimmune gastritis, psoriasis, Bechet's disease, Wegener's granulomatosis, sarcoidois, autoimmune thyroiditis, autoimmune oophoritis, bullous pemphigoid, phemphigus, polyendocrinopathies, Still's disease, Lambert-Eaton myasthenia syndrome, myasthenia gravis, Goodpasture's syndrome, autoimmune orchitis, autoimmune uveitis, systemic lupus erythematosus, Sjogren's Syndrome and ankylosing spondylitis, as well as any other disorder or syndrome now known or later identified to be autoimmune disease.

As used herein, "graft-versus-host" (GvH) disease describes a disease state or syndrome whereby an immune response is initiated by grafted cells and is directed against the subject's body with adverse effects. Examples of GvH disease include, but are not limited to, acute and chronic GvH disease following bone marrow transplant.

As used herein, "transplantation rejection" describes a disease state or syndrome whereby the transplant recipient's body produces an immune response against the engrafted tissue, resulting in rejection. Transplantation rejection can occur, for example, with bone marrow, kidney, heart, lung or liver transplants as well as with any other transplanted tissue.

Further provided by the present invention is a method for increasing IL-12 production in a subject, comprising administering an effective amount of a CCR5 agonist to the subject. Increased IL-12 production may be desirable when a subject has an infectious disease, an atopic/allergic condition or cancer.

Examples of infectious disease include, but are not limited to, tuberculosis, listeriosis, histoplasmosis, candidiais, schistosomiasis, as well as any other disease transmitted by an infectious agent which depends on Th1 responses driven by IL-12 that is now known or later identified.

As used herein, "atopic or allergic" describes a disease state or syndrome whereby the body produces a dysfunctional immune response composed of immunoglobulin E (IgE) antibodies due to either a genetic predisposition or exposure to environmental antigens and wherein allergic symptoms are manifested. Examples of atopic/allergic diseases include, but are not limited to, asthma, ragweed pollen hay fever, allergy to food substances, atopic eczema, hypersensitivity pneumonitis, Farmers lung, hypereosinophilic syndromes and allergic reactions.

The terms "cancer," and "cancerous" when used herein refer to or describe the physiological condition, generally in a mammalian subject, that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, melanoma, sarcoma, blastoma and leukemia. More particular examples of such cancers include squamous cell carcinoma, lung cancer, pancreatic cancer, cervical cancer, bladder cancer, hepatoma, breast cancer, prostrate carcinoma, rhabdomyosarcoma, renal cancer, colon carcinoma, and head and neck cancer. While the term "cancer" as used herein is not limited to any one specific form of the disease, it is believed that the methods for increasing IL-12 production of this invention will be particularly effective for effecting cell-mediated immunity against malignancies or to promote Th1 responses protective against tumors.

In the treatment of cancer, the CCR5 agonist may also be administered in combination with effective amounts of one or more other therapeutic agents or in conjunction with radiation treatment. Therapeutic agents contemplated include chemotherapeutics as well as immunoadjuvants and cytokines. Chemotherapies contemplated by the invention include chemical substances or drugs which are known in the art and are commercially available, such as Doxorubicin, 5-Fluorouracil, Cytosine arabinoside ("Ara-C"), Cyclophosphamide, Thiotepa, Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine and Carboplatin. The CCR5 agonist may be administered sequentially or concurrently with the one or more other therapeutic agents. The amounts of CCR5 agonist and therapeutic agent depend, for example, on what type of drugs are used, the condition being treated, and the scheduling and routes of administration but would generally be less than if each were used individually. Following administration of CCR5, the condition can be monitored in various ways well known to the skilled practitioner. For instance, tumor mass may be observed physically or by standard x-ray imaging techniques.

In the present invention, the subject can be any animal capable of expressing CCR5. The subject of this invention can be a mammal, which can be, for example, a mouse, rat, guinea pig, hamster, rabbit, cat, dog, goat, monkey, horse or chimpanzee, although in a preferred embodiment, the subject of this invention is a human.

As used herein, "treating or treatment" describes an improvement in, or modulation of, the subject's clinical state as a result of administration of the agonists or antagonists of the present invention. The improvement or modulation can be, for example, a reduction in the severity of symptoms of the disease, the elimination of symptoms of the disease, a reduction in the disease response and/or complete amelioration of the disease state. The improvement or modulation in the subject is determined on the basis of clinical parameters well known to the clinician for a given disease state.

In the present invention, a CCR5 antagonist or CCR5 agonist can be orally or parenterally administered to a subject in a pharmaceutically acceptable carrier. The CCR5 antagonist or CCR5 agonist of this invention can also be administered in an enema, or as an inhalant, nasal spray or eye drops. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject along with agonist or antagonist without causing any substantial undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. Actual methods of preparing dosage forms are known, or will be apparent, to those skilled in this art (see, e.g., Martin, latest edition; Arnon, 1987). The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

Suitable carriers for oral or inhaled administration of the CCR5 antagonist or CCR5 agonist can include one or more of the carriers pharmaceutically acceptable to human subjects. Suitable carriers for oral administration of the CCR5 antagonist or CCR5 agonist include one or more substances which may also act as a flavoring agents, lubricants, suspending agents, or as protectants. Suitable solid carriers include calcium phosphate, calcium carbonate, magnesium stearate, sugars, starch, gelatin, cellulose, carboxypolymethylene, or cyclodextrans. Suitable liquid carriers may be water, pyrogen free saline, pharmaceutically accepted oils, or a mixture of any of these. The liquid can also contain other suitable pharmaceutical addition such as buffers, preservatives, flavoring agents, viscosity or osmo-regulators, stabilizers or suspending agents. Examples of suitable liquid carriers include water with or without various additives, including carboxypolymethylene as a pH-regulated gel. The antagonist or agonist may be contained in enteric coated capsules that release the antagonist or agonist into the intestine to avoid gastric breakdown.

For parenteral administration of the antagonist or the agonist, a sterile solution or suspension is prepared in saline that may contain additives, such as ethyl oleate or isopropyl myristate, and can be injected for example, into subcutaneous or

intramuscular tissues, as well as intravenously. A CCR5 antagonist or agonist may be contained in enteric coated capsules that release the inhibitor into the intestine to avoid gastric breakdown.

Alternatively, a CCR5 antagonist or CCR5 agonist may be microencapsulated with either a natural or a synthetic polymer into microparticles 4-8 μm in diameter, which target intestinal lymphoid tissues and produce a sustained release of inhibitor for up to four weeks.

As examples of administration protocols for the CCR5 antagonists or CCR5 agonists of this invention, the CCR5 antagonists or CCR5 agonists, in soluble form, can be administered to a subject, parenterally in a single dosage or multiple dosages ranging from about 0.1 mg to about 100 mg/kg of body weight, with a preferred dosage range of about 1-50 mg/kg and a most preferred dosage range of about 2-10 mg/kg. Subjects can be given CCR5 antagonists or CCR5 agonists as a single injection approximately weekly for an indefinite period, as determined by the subject's response.

For oral administration, about 500 mg to about 1000 mg of CCR5 antagonists or CCR5 agonists can be given PO. For administration of CCR5 antagonists or CCR5 agonists in particulate form, about 500 mg to about 1000 mg can be microencapsulated as described for slow release over a four to eight week period. The treatment can be reinitiated at any time, particularly in the event of a recurrence. Depending on the particular disease and the patient's condition, a maintenance dosage may be administered to prevent recurrence. This dosage can be administered every other week to monthly for an indefinite period, depending on the nature of the disease associated with increased or decreased IL-12 production, which may vary from an acute condition to a maintenance state.

The amount and combination of CCR5 antagonists or CCR5 agonists administered will vary among individuals based on the age, size, weight and/or overall condition of the subject, as well as the particular disorder being treated, severity of the

disorder, etc. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods for determining dosage are described, for example in Remington's Pharmaceutical Science (17).

The efficacy of administration of a particular dose of CCR5 antagonists in a subject diagnosed as having an autoimmune disease, inflammatory disease or GvH disease can be determined by standard methods of evaluation of the particular signs, symptoms and objective laboratory tests for a particular disease, as are well known in the art.

The efficacy of administration of a particular dose of CCR5 agonists in a subject diagnosed with an infectious disease, asthma, allergic/atopic condition or cancer can be determined in a similar fashion. For example, if 1) a subject's frequency or severity of recurrences is shown to be improved, 2) the progression of the disease is shown to be stabilized, or 3) the need for use of other medications is lessened, based on a comparison with an appropriate control group and knowledge of the normal progression of disease in the general population or the particular individual, then a particular treatment is considered efficacious.

Also contemplated by this invention is a method for preventing disease by administering a CCR5 agonist, in either protein or recombinant form as an adjuvant or immunostimulant in conjunction with a vaccine or foreign antigen as a means of enhancing IL-12 production in order to enhance the development of protective cell-mediated (Th1) responses against the antigen and suppress deleterious Th2 responses induced by an infectious disease, environmental stimulus or cancer against which the vaccine is meant to protect. Upon exposure to the agent (pathogen, allergen or cancer) individuals vaccinated in this manner would be more immune than those receiving a vaccine lacking the CCR5 agonist component. One variation of the approach is to vaccinate in this manner in order to suppress Th2 cell dependent inflammation induced by a subsequent infection or allergic exposure (18, 19). In this embodiment, the vaccine is not preventing infection or allergic contact but deviating the response to a

Th1 response so that it is not harmful. By incorporating CCR5 agonists in a vaccine, one would increase the resistance it induces against infections and tumors and/or promote its ability to block allergic or pathogen induced inflammation.

In addition to treating the diseases of this invention, the CCR5 agonists of this invention can be used in preventing the onset of disease in a subject who is at risk for the development of an infectious disease, allergic/atopic disease or cancer.

The determination of who would be at risk for the development of an infectious disease, allergic/atopic disease or cancer would be made based on current knowledge of the known risk factors for a particular disease or cancer familiar to a clinician in this field, such as a particularly strong family history of disease or cancer, an established genetic predisposition for a particular disease or cancer and/or exposure to or a connection with specific environmental factors associated with a particular disease or cancer. Thus, the present invention further provides a method of preventing an infectious disease, allergic/atopic disease or cancer in a subject, comprising administering an effective amount of a CCR5 agonist to the subject.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

An *in vivo* model system for studying pathogen-induced DC activation involving the intravenous injection into mice of an extract (STAg) of tachyzoites of *Toxoplasma gondii* was established (3- 4). This opportunistic protozoan is known to induce a potent IL-12 response early in infection that results in interferon (IFN)-γ dependent control of parasite growth (5). Within several hours after STAg injection, splenic CD8α^+ DC produce high levels of IL-12p40 and upregulate their expression of costimulatory molecules (6). This response occurs as the DC migrate from the red pulp

and marginal zones of the spleen into the inner T cell region of the periarteriolar lymphoid sheath (PALS). In order to study the role of chemokines in this mobilization, the induction of chemokine mRNAs in spleen after STAg injection was measured. As early as 1 hr after *in vivo* stimulation, chemokine messages were detected at high levels in splenic extracts. mRNAs for CCR5 ligands were abundantly represented and immunofluorescent staining confirmed the expression of MIP-1α and MIP-1β protein in response to STAg inoculation. Interestingly, these CCR5 ligands localized to the central arterioles of the spleen suggesting endothelial cells as a likely source and providing a possible explanation for the centripetal directional movement of DC within PALS.

To evaluate the role of CCR5 ligands in DC migration, a CCR5 antagonist, Met-RANTES (7) was administered to mice before injection of STAg. *In vivo* treatment with Met-RANTES resulted in partially impaired STAg-induced DC mobilization, as evidenced by the formation of DC clusters which were more diffuse than those observed in untreated STAg-injected mice. A nearly identical pattern of defective DC clustering was observed when mice genetically deficient in CCR5 were tested in the same assay.

Unexpectedly, spleen sections from STAg-injected Met-RANTES-treated or CCR5-deficient mice showed diminished immunoreactivity with anti-IL-12p40 mAb. This reduction in IL-12 expression was confirmed by intracellular staining of splenic $CD11c^{+}CD8\alpha^{+}$ DC for the cytokine (Fig. 1A) as well as by ELISA measurements performed on supernatants of cultured splenocytes from the drug-treated animals (Fig. 1C). Consistent with its weaker affinity for murine CCR5, human RANTES failed to significantly inhibit STAg-induced IL-12 production *in vivo*, (Fig. 1C). Moreover, STAg-injected CCR5-deficient mice showed even greater (83-89%) defects in splenic IL-12 responses than Met-RANTES-treated mice. The reductions in IL-12 expression observed were comparable in the two different read-outs of DC cytokine production performed (Fig. 1B and 1D).

To investigate the mechanism by which microbial products stimulate CCR5-dependent IL-12 production spleen cells were exposed *in vitro* to STAg in the presence of Met-RANTES. As shown in Figure 2A, a dose-dependent inhibition of IL-12 production was observed, but this suppression was partial (maximum = 45%). A similar (54%) loss in activity was observed when CCR5 deficient splenocytes were compared in the same assay with wild type cells (Fig. 2B). Nevertheless, $CD8\alpha^-$ DC purified from the CCR5 deficient spleens displayed a greater (72%) defect in cytokine production (Fig. 2C), suggesting that in this subset of DC, CCR5 is a major receptor for IL-12 stimulation. The residual non-CCR5 dependent IL-12 induction may involve other chemokine receptors since *Pertussis* toxin, a reagent which uncouples the G-protein signaling pathway used by all chemokine receptors, causes a near total inhibition in STAg-induced IL-12 production (Fig. 2D).

To confirm that CCR5/ligand interaction can directly lead to IL-12 expression by DC, the ability of recombinant CCR5 chemokines to induce IL-12 p40 synthesis by spleen cells and purified $CD8\alpha^+$ DC *in vitro* was assessed. As shown in Figure 3A, MIP-1β stimulated a highly significant IL-12p40 response (measured by ELISA) in wild type, but not CCR5 deficient splenocyte cultures. When FACS sorted DC populations were tested in the same assay, $CD8\alpha^+$ but not $CD8\alpha^-$ DC, responded to MIP-1β stimulation (Fig. 3B). Consistent with the latter observation, $CD8\alpha^-$ but not $CD8\alpha^+$ DC were found to bind FITC-labeled MIP-1β. A comparison of different recombinant chemokines revealed that amongst the murine CCR5 ligands tested, MIP-1β and RANTES induced the highest levels of IL-12 production while MIP-1α stimulated a response only at high concentrations. In contrast, human MIP-5 and the murine CCR2 ligand MCP-1 (obtained from the same commercial source as the MIP-1β) failed to display significant IL-12-inducing activity (Fig. 3C). As expected, *Pertussis* toxin completely inhibited IL-12 induction by MIP-1β, indicating that the stimulation of cytokine synthesis occurs through the same signaling pathway as that responsible for triggering chemotaxis (Fig 3D).

The above findings establish CCR5 as a receptor that can trigger IL-12 production by the CD8α⁻ subset of DC and demonstrate a role for this pathway in microbial stimulation of IL-12 synthesis. In addition to this mechanism, STAg itself may contain proteins with motifs that directly bind to and stimulate CCR5 as suggested by its ability to induce IL-12 production by purified CD8α⁻DC *in vitro* (Fig. 2C).

In vivo microbial stimulation

C57Bl/6, B6129F2/J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and CCR5 deficient mice were obtained from the breeding unit of University of Michigan (Ann Arbor, MI). Soluble tachyzoite antigen (STAg) was prepared from tachyzoites of the RH 88 strain of *T. gondii,* as previously described (14). STAg was administered to mice i.p. at a dose of 20 μg per mouse diluted in 0.2 ml of PBS. Control mice received the same volume of PBS.

In situ staining of spleen cell sections

Spleens were removed from mice 6 hr after microbial stimulation, and frozen sections (8-10 μm) cut, processed and stained with antibodies specific for IL-12p40 or the dendritic cell marker CD11c, as previously described (3). For *in situ* detection of chemokines, a two step method was used, involving primary incubation with a goat anti-mouse MIP-1α or anti-MIP-1β polyclonal antibodies (Santa Cruz Biotech., Santa Cruz, CA), followed by a second incubation with FITC-conjugated anti-goat antibody (Santa Cruz) and PE-conjugated anti-mouse CD11c (Pharmingen, San Diego, CA) for 30 min.

Measurement of IL-12 production by spleen cells and isolated DC populations

Spleen cells suspensions were obtained by collagenase D digestion and the low-density leukocyte (LOD) fraction prepared by centrifugation on a dense BSA gradient, as previously described (3). For intracellular IL-12p40 staining, spleen cell suspensions were first incubated with antibodies against CD11c (FITC) and CD8α (PE) (Pharmingen), fixed for 20 minutes in Cytofix/Cytoperm solution (Pharmingen), followed by a 30 min incubation with an APC-labeled anti-IL-12p40 Ab (Pharmingen).

200,000 events were collected on a FACS Calibur cytometer (Becton-Dickinson) and analyzed with FlowJo software (Treestar).

To study IL-12 responses in purified DC populations, LOD cells were stained with FITC-anti-CD11c and PE-anti-CD8α Ab and $CD11c^+CD8\alpha^-$ and $CD11c^+CD8\alpha^+$ cell subsets were sorted in a FACS Vantage (Becton-Dickinson). Purity of DC subpopulations after sorting was 97±1% for both subsets. To measure secreted IL-12, spleen cell suspensions, LOD fractions, or sorted cells were plated into 96-well microplates and stimulated with STAg (2 μg/ml), or the recombinant chemokines: MIP-1α, RANTES, MIP-5 (Peprotech), MIP-1β and MCP-1 (R&D Systems) for 24 hr at 37°C. The supernatants were then harvested and assayed for IL-12p40 using a sandwich ELISA as previously described (15).

This invention formally demonstrates that chemokine/receptor interaction can lead to cytokine induction and identifies CCR5 as a receptor that selectively triggers IL-12 in the $CD8\alpha^+$ subset of DC. The present invention demonstrates that signaling through the CC chemokine receptor (CCR)5 promotes microbial-induced DC migration as well as IL-12 production by the $CD8\alpha^+$ subset of these cells present in spleen. Moreover, the invention shows that recombinant CCR5 ligands stimulate substantial IL-12 responses *in vitro* by $CD8\alpha^-$, but not $CD8\alpha^+$ DC purified directly from spleen. These findings show that chemokine-receptor interactions are important upstream events in both the recruitment and activation of DC by pathogens and demonstrate that in addition to their well-established functions in cell migration, chemokine receptors can play a role in triggering cytokine synthesis.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties, as well as the references cited in these publications, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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What is claimed is:

1. A method for increasing IL-12 production in a cell, comprising administering a CCR5 agonist to the cell.

2. The method of claim 1, wherein the cell is a dendritic cell.

3. The method of claim 1, wherein the CCR5 agonist is selected from the group consisting of MIP-1α, MIP-1β, MIP-5, and MCP-3.

4. A method for decreasing IL-12 production in a cell comprising administering a CCR5 antagonist to the cell.

5. The method of claim 4, wherein the cell is a dendritic cell.

6. The method of claim 4, wherein the CCR5 antagonist is selected from the group consisting of LD-478, viral MIP-II, and neutralizing CCR5monoclonal antibodies.

7. A method for treating a disease associated with increased IL-12 production in a subject comprising administering to the subject a CCR5 antagonist in an amount effective in reducing the disease-associated effect of IL-12 thereby treating the disease associated with increased IL-12 production.

8. The method of claim 7, wherein the disease associated with increased IL-12 production is an autoimmune disease.

9. The method of claim 7, wherein the autoimmune disease is selected from the group consisting of ulcerative colitis, Crohn's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, pernicious anemia, autoimmune gastritis,

psoriasis, Bechet's disease, Wegener's granulomatosis, Sarcoidois, autoimmune thyroiditis, autoimmune oophoritis, bullous pemphigoid, phemphigus, polyendocrinopathies, Still's disease, Lambert-Eaton myasthenia syndrome, myasthenia gravis, Goodpasture's syndrome, autoimmune orchitis, autoimmune uveitis, systemic lupus erythematosus, Sjogren's Syndrome and ankylosing spondylitis.

10. The method of claim 7, wherein the disease associated with increased IL-12 production is an inflammatory disease.

11. The method of claim 7, wherein the disease associated with increased IL-12 production is graft-versus-host disease.

12. A method for increasing IL-12 production in a subject, comprising administering an effective amount of a CCR5 agonist to the subject.

13. The method of claim 12, wherein the subject has an infectious disease.

14. The method of claim 12, wherein the subject has an atopic condition.

15. The method of claim 12, wherein the subject has cancer.

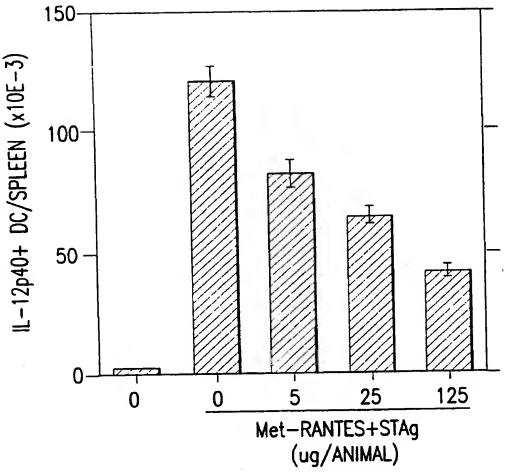

FIG.1A

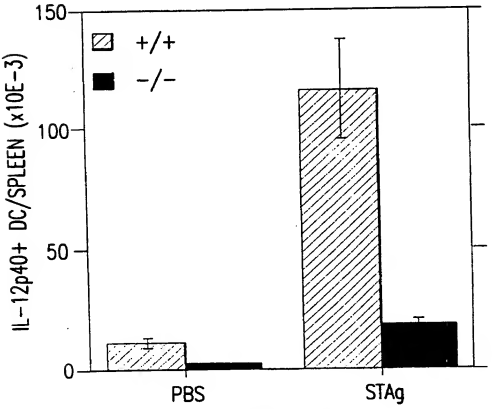

FIG.1B

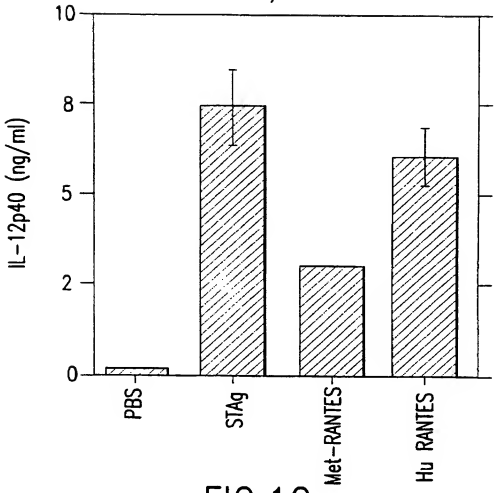

FIG.1C

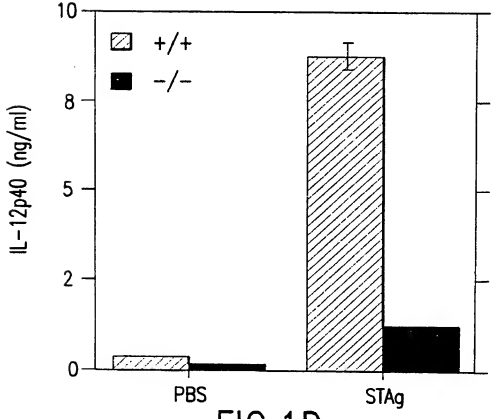

FIG.1D

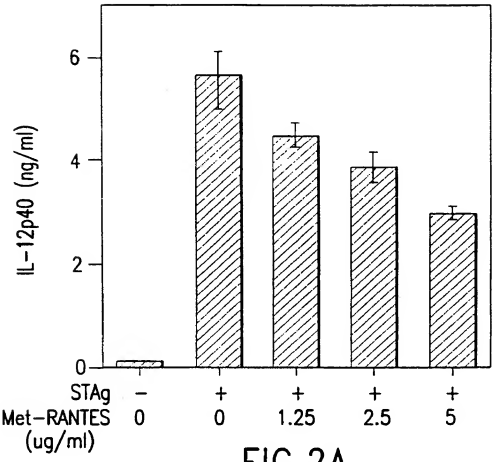

FIG.2A

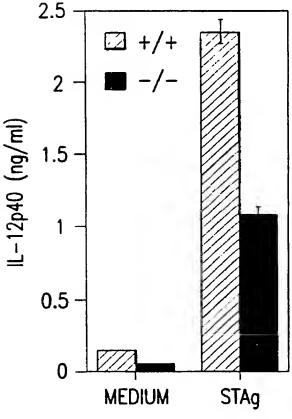

FIG.2B

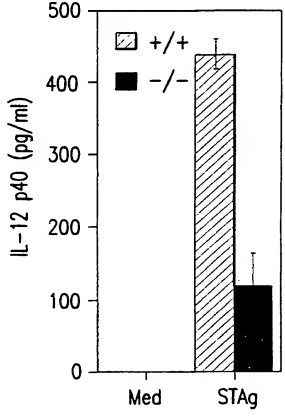

FIG.2C

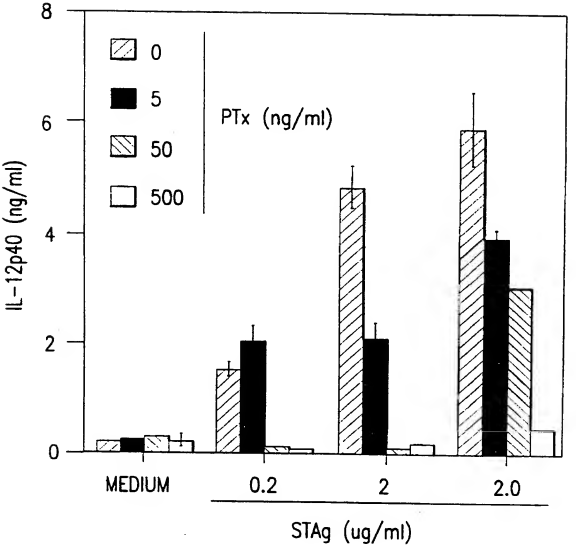

FIG.2D

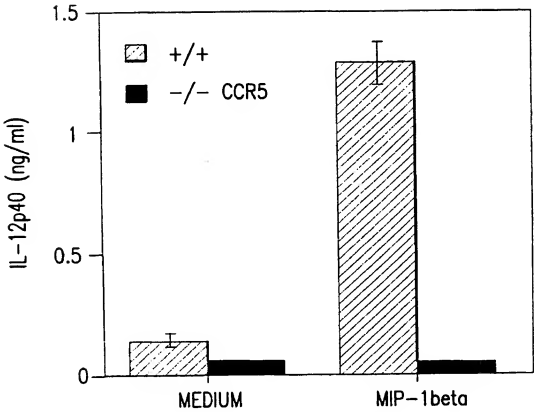

FIG.3A

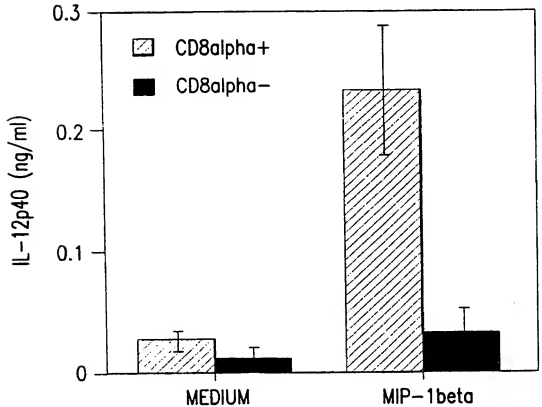

FIG.3B

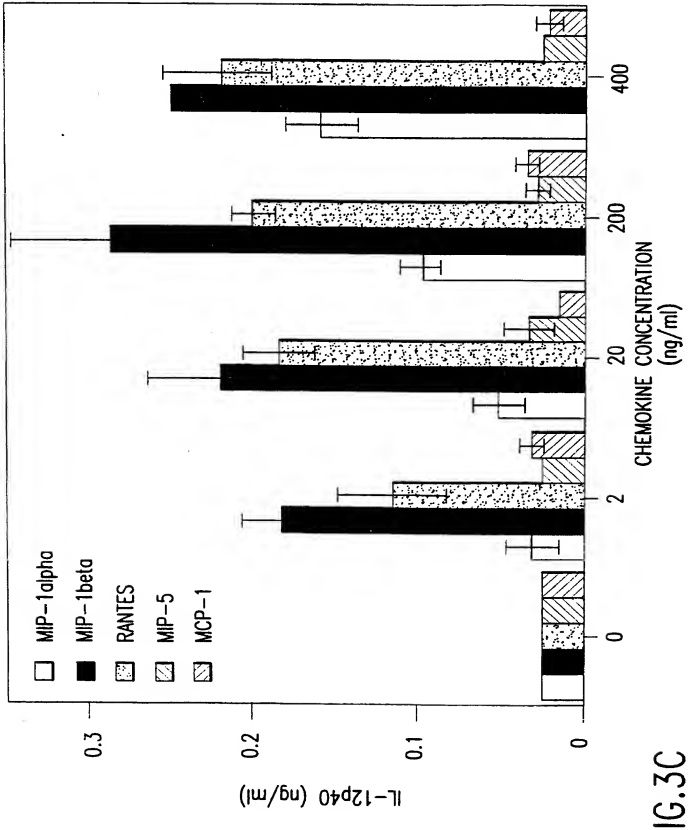

FIG.3C

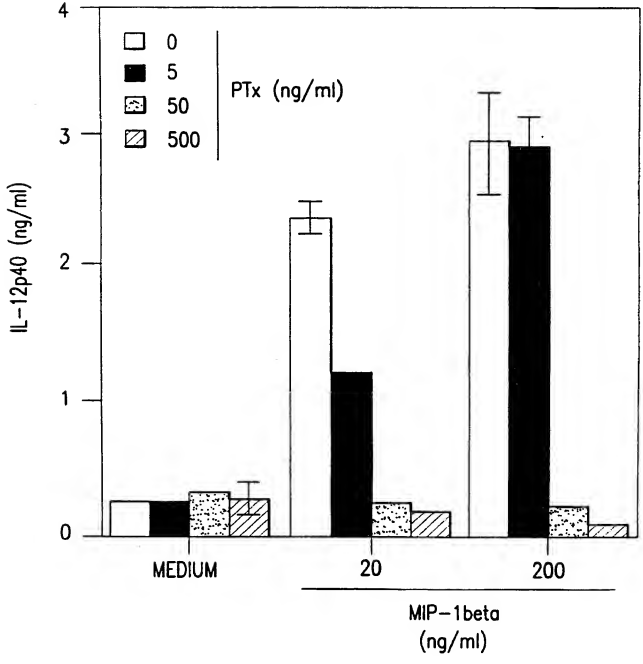

FIG.3D

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/US 00/01019

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/19 A61K39/395 A61P37/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, CANCERLIT, AIDSLINE, LIFESCIENCES, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OLSZEWSKI M A ET AL: "The role of MIP-1alpha in the regulation of Th1 versus Th2 immune responses to Cryptococcus neoformans." ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 99, 1999, page 303 XP002149557 99th General Meeting of the American Society for Microbiology; Chicago, IL, USA; May 30-June 3, 1999, 1999 abastract F-38 --- -/--	1-3, 12-15

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2000	24 10 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Teyssier, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
X	SILVA J S ET AL: "Cytokines, chemokines, and apoptosis in the regulation of immune response to Trypanosoma cruzi." MEMORIAS DO INSTITUTO OSWALDO CRUZ, vol. 94, no. Supplement 2, November 1999 (1999-11), pages 35-36, XP000952588 XXVI Annual Meeting on Basic Research in Chagas' Disease and the XV Annual Meeting of Brazilian Society of Protozoology.; Caxambu, Brazil; November 09-11, 1999 the whole document	4-11
A	SHER A & REIS E SOUSA C: "Ignition of the type I response to intracellular infection by dendritic cell-derived interleukin-12." EUROPEAN CYTOKINE NETWORK, vol. 9, no. SUPPL. 3, 1998, pages 65-68, XP000952573 cited in the application	
A	SATO N ET AL: "Defects in generation of IFN-gamma are overcome to control infection with Leshmania donovani in CC chemokine receptor (CCR) 5-, Macrophage Inflammatory Protein-1alpha-, or CCR2-deficient mice" JOURNAL OF IMMUNOLOGY, vol. 163, no. 10, 15 November 1999 (1999-11-15), pages 5519-5525, XP002149558 cited in the application	
A	ALIBERTI J C S ET AL: "Beta-chemokines enhance parasite uptake and promote nitric oxide-dependent microbiostatic activity in murine inflammatory macrophages infected with Trypanosoma cruzi." INFECTION AND IMMUNITY, vol. 67, no. 9, September 1999 (1999-09), pages 4819-4826, XP002149559	
A	WANG J ET AL: "Inhibition of CCR5 expression by IL - 12 through induction of beta - chemokines in human T lymphocytes." JOURNAL OF IMMUNOLOGY, vol. 163, no. 11, 1 December 1999 (1999-12-01), pages 5763-5769, XP002149560	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
T	COLLAZO C M ET AL: "Host resistance and immune deviation in pigeon cytochrome c T-cell receptor transgenic mice infected with Toxoplasma gondii." INFECTION AND IMMUNITY, vol. 68, no. 5, May 2000 (2000-05), pages 2713-2719, XP002149561	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/01019

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although all claims are directed to methods of treatment of the human body, the search has been carried out and based on the alleged effects of the compounds.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.